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Abstract

Mutations in the DMD gene lead to Duchenne and Becker muscular dystrophy (DMD/BMD). Missense mutations are rare cause of DMD/BMD. A six-month-old male patient presented with mild generalized muscle weakness, hypotonia, and delayed motor development. Dystrophinopathy was suspected because of highly elevated serum creatine kinase level (1497 U/L) and tiered DMD gene analysis was performed. Multiplex ligation-dependent probe amplification (MLPA) assay showed deletion of exon 4, which could not be confirmed by another method. Sequencing of exon 4 revealed a novel de novo point mutation (c.227A>T, p.Asn76Ile) in the N-terminal actin binding domain (N-ABD) of dystrophin protein. The false positive MLPA result was explained by the fact that the affected nucleotide lies directly at the 3' ligation site of the MLPA probe. Sequencing of the whole coding region of DMD gene proved c.227A>T to be the sole variant being potentially pathogenic. According to in silico analyses the mutation was predicted to be highly destabilizing on N-ABD structure possibly leading to protein malfunction. Muscle biopsy was performed and dystrophin immunohistochemistry results were suggestive of BMD. Our results highlight the importance of confirmatory testing of single-exon deletions detected by MLPA and we describe a novel, destabilizing missense mutation in the DMD gene.

Keywords	Duchenne/Becker muscular dystrophy, MLPA, dystrophin, dystrophinopathy

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Prof. Dr. Victor Dubowitz
Editor-in-Chief
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Dear Professor Dubowitz,

Enclosed please, find our manuscript, entitled *"A novel point mutation affecting Asn76 of dystrophin protein leads to dystrophinopathy"* which we would like to publish in *Neuromuscular Disorders*.

In the paper we present a comprehensive analysis of a very young patient with dystrophinopathy. Using different methods, including MLPA, next generation and Sanger sequencing we show the presence of a novel point mutation (c.227A>T, p.Asn76Ile) that is located in a critical part of the protein involved in actin binding. Using *in silico* methods we show that the mutation might have destabilizing effect which fact is supported by immunohistochemistry analysis.

We believe that the complexity of the presented case and the use of state-of-the-art methodology (i.e. very young age of the patient, false-positive MLPA analysis, identification of a novel missense mutation, next-generation sequencing of the entire coding region of the gene) might be interesting for a broader audience.

The detected novel mutation (LRG_199t1: c.227A>T, LRG_199p1: p.Asn76Ile) has been submitted to the LOVD database (https://databases.lovd.nl/shared/variants/0000171133).

We confirm that

- all listed authors have actively participated in the study,
- all authors have seen and approved the manuscript,
- the study complies with the current ethical considerations,
- the manuscript reports previously unpublished work that will not be submitted to any other journal whilst it is under consideration by *Neuromuscular Disorders*,
- the authors have no conflict of interest.

Debrecen, 2017. 06. 27

Thank you for your consideration.

Yours sincerely,

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A novel point mutation affecting Asn76 of dystrophin protein leads to dystrophinopathy

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Highlights

- ~~1.~~ Missense mutations are rare cause of Duchenne/Becker muscular dystrophy and the major fraction of them occurs in the N-ABD of dystrophin protein.
- ~~2.~~ Our results highlight the importance of confirmatory testing of single-exon deletions detected by MLPA.
- ~~3.~~ To predict the severity of the phenotype may be challenging at very young age in the case of a novel missense mutation in the *DMD* gene.

Title page

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Conflicts of interest: none.

Abstract

Mutations in the *DMD* gene lead to Duchenne and Becker muscular dystrophy (DMD/BMD). Missense mutations are rare cause of DMD/BMD. A six-month-old male patient presented with mild generalized muscle weakness, hypotonia, and delayed motor development. Dystrophinopathy was suspected because of highly elevated serum creatine kinase level (1497 U/L) and tiered *DMD* gene analysis was performed. Multiplex ligation-dependent probe amplification (MLPA) assay showed deletion of exon 4, which could not be confirmed by another method. Sequencing of exon 4 revealed a novel *de novo* point mutation (c.227A>T, p.Asn76Ile) in the N-terminal actin binding domain (N-ABD) of dystrophin protein. The false positive MLPA result was explained by the fact that the affected nucleotide lies directly at the 3' ligation site of the MLPA probe. Sequencing of the whole coding region of *DMD* gene proved c.227A>T to be the sole variant being potentially pathogenic. According to *in silico* analyses the mutation was predicted to be highly destabilizing on N-ABD structure possibly leading to protein malfunction. Muscle biopsy was performed and dystrophin immunohistochemistry results were suggestive of BMD. Our results highlight the importance of confirmatory testing of single-exon deletions detected by MLPA and we describe a novel, destabilizing missense mutation in the *DMD* gene.

Keywords

Duchenne/Becker muscular dystrophy, MLPA, dystrophin, dystrophinopathy

[1]1. Introduction

Mutations in the *DMD* gene (Xp21.2-p21.1) encoding the protein dystrophin lead to the X-linked allelic disorders Duchenne (DMD, MIM 310200) and Becker muscular dystrophy (BMD, MIM 300376), both characterized by progressive muscle wasting [1, 2]. DMD is the more severe form caused by mutations that result in nearly complete absence of dystrophin protein [3, 4]. BMD is the milder variant characterized by remarkable variability in presentation and progression of symptoms and associated with abnormal but partially functional dystrophin protein [5, 6]. Dystrophin is associated with the sarcolemma and interacts with integral membrane proteins assembled in the dystrophin-glycoprotein complex forming a link between the extracellular matrix and the cytoskeletal actin. Dystrophin's main role is the stabilization of sarcolemma and the protection of muscle fibers from contraction-induced damage [7]. Several isoforms of the protein are generated through different promoters and alternative splicing events. The full-length messenger RNA is predominantly expressed in skeletal and cardiac muscle and, to a lesser extent, in the brain [8]. The full-length human dystrophin protein transcribed from the muscle promoter consists of 3685 amino acids (molecular weight of 427 kDa) [1] and is predicted to fold into four structural domains: the N-terminal actin-binding domain (N-ABD or ABD1), a central rod domain (containing a second ABD, ABD2), a cysteine-rich domain and a C-terminal domain [9].

Clinical diagnosis of DMD/BMD based on elevated serum creatine kinase (CK) and/or muscle biopsy can be confirmed by molecular genetic testing of the *DMD* gene. The first tier

[1] **Abbreviations:** *DMD,* dystrophin gene; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; N-ABD, N-terminal actin binding domain; CK, creatine kinase; MLPA, multiplex ligation-dependent probe amplification; dbSNP, single nucleotide polymorphism database; MAF, minor allele frequency; ABS, actin binding site; ExAC, Exome Aggregation Consortium.

genetic analysis is screening for deletions/duplications, which account for about 75-85% of genetic alterations in DMD/BMD patients [10, 11]. If no deletion or duplication is found, sequencing of the *DMD* gene as a second tier test can be offered [12]. The great majority of point mutations in the *DMD* gene so far identified are nonsense mutations, small frameshift deletions/insertions and splice-site mutations [8], missense mutations are rare cause of DMD/BMD.

The *DMD* gene is one of the largest human genes spanning more than 2.5 million base pairs and consists of 79 exons [8]. The high mutation rate of the gene has been claimed to be the consequence of very large introns [13], in one of three patients the disease is the result of a *de novo* mutation [14]. Germline mosaicism is a significant contributing factor to introduce *DMD* gene mutations [15] and has to be taken into account when considering recurrence risk [16].

Structural elements that are responsible for the function of dystrophin (*i.e.* maintaining the link between actin and the extracellular matrix) are well characterized. An antiparallel dimer is formed by the N-ABDs of two dystrophin molecules, which can be seen in the crystal structure on human dystrophin protein [17] (Figure 5A). The N-ABD of human dystrophin is mainly helical and contains two calponin homology (CH) domains. Its CH1 and CH2 subdomains are connected by a helical linker region (helix I) (Figure 5A). CH2 subdomain contains major (A, C, E, and G) and short (B, D, and F) helices. Overall fold of CH1 closely resembles that of CH2 but lacks the short B and D helices, which are substituted by loop regions in this subdomain (Figure 5A). Three actin-binding sites are responsible for interactions with actin molecules. The ABS1 (K18-A27) is located in helix A while the ABS2 (N88-L116) mainly in F and G helices of CH1 subdomain. The ABS3 site (L131-S147) is located in helix A of CH2 subdomain.

Here we report the detection of a novel missense mutation located in the N-ABD of

dystrophin protein affecting Asn76 residue. Interpretation of this novel genetic finding is given in the context of clinical picture, muscle biopsy analysis, and *in silico* analysis of protein structure.

2. Patients and methods

2.1 Case history

A six-month old male patient was sent for neurologic examination because of mild generalized muscle weakness, hypotonia, and delayed motor development. Perinatal anamnesis, mental development, and sensation were found to be normal normal. As in spite of sensory-motor training no improvement of symptoms was observed, a neuromuscular disease was suspected. Electromyography showed myopathic features (data not shown). Laboratory investigation at 1-year-old age showed markedly elevated serum creatine kinase (1497 U/L, reference range: 24-195 U/L) prompting the consideration of dystrophinopathy and *DMD* gene analysis was requested at age of 14 months. There was no family history of DMD/BMD. Follow-up physical examination revealed difficulties in sitting up from lying, scapula alata, decreased muscle bulk on upper extremities, and chest in addition to mild calf hypertrophy at the age of 4 years. Changes in serum creatine kinase values during follow-up time are shown in Figure 1.

2.2 Molecular genetic analysis

2.2.1 DNA isolation

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

2.2.2 Deletion/duplication analysis of *DMD* gene

Multiplex ligation-dependent probe amplification (MLPA) analysis for the detection of deletions/duplications in the *DMD* gene was performed using the SALSA MLPA probe mixes P034-B2 DMD and P035-B1 DMD (MRC-Holland, Amsterdam, the Netherlands). Data were analyzed according to the manufacturer's instructions. Briefly, relative peak heights were generated after intra- and inter-sample normalization using sex-matched control samples. Deletion of the probe's recognition sequence leads to complete absence of the corresponding probe amplification product in males.

2.2.3 Sanger DNA sequencing of exon 4 of *DMD* gene

After PCR amplification of the coding sequence and adjacent intronic regions of exon 4 of *DMD* gene bidirectional Sanger DNA sequencing was performed using amplification primers from the Chamberlain set [18] and the Big Dye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions. Samples were run on ABI PRISM 310 Genetic Analyzer and data were analyzed using the Sequencing Analysis Software (Applied Biosystems, Foster City, CA, USA).

2.2.4 Sequencing of the entire coding region of *DMD* gene

Sequencing of *DMD* gene was performed by bidirectional pyrosequencing on GS Junior system using the DMD MASTR assay (Multiplicom, Niel, Belgium). Data were analyzed with the GS Amplicon Variant Analyzer software (Roche 454 Life Sciences, Branford, CT, USA).

2.3 Muscle histology, dystrophin immunohistochemistry

The muscle biopsy specimen measuring 0,5x0.4x0,8 cm from left quadriceps muscle was divided into three equal parts and i) snap frozen in isopentane for histology and immunohistochemistry; ii) fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) for histology; iii) fixed in 3% phosphate-buffered glutaraldehyde (pH 7.4) to ensure tissue was available for electronmicroscopy if required (which was not the case here, because light microscopic and genetic analysis were sufficient to provide the diagnosis). Frozen sections of 7 micrometer thickness were cut for detailed muscle biopsy analysis. Staining, enzyme- and immunohistochemistry was performed according to diagnostic guidelines and standard protocols. In brief, sections were stained by haematoxylin and eosin (H&E) for general neuropathological analysis, by periodic-acid Schiff reaction (PAS) with and without diastase treatment for assessment of glycogen, with Sudan-black for lipids, with Gömöri's trichrome for assessment of mitochondrial pathology, fibrosis, and inclusions. Fiber type analysis was performed following ATPase detection by enzyme histochemistry at pH 4.2, 4.6, and 9.4, respectively. Mitochondrial function and myofibrillary architecture was assessed following succinate dehydrogenase (SDH), nicotinamide adenine dinucleotide dehydrogenase (NADH), and cytochrome oxidase (COX) enzyme histochemistry. Immunohistochemistry was performed by using antibodies against sarcolemmal and sarcolemma-associated proteins: dystrophin targeting epitopes 1, 2, and 3, corresponding to central/core, C-terminal and N-terminal regions, respectively, (Novocastra, Newcastle, UK, distributed by Biomarker, Gödöllő, Hungary; primary antibody dilution was 1:20 in all cases), sarcoglycan alpha, beta, gamma, and delta (Novocastra, 1:50, 1:100, 1:100, and 1:50, respectively); merosin (Novocastra, 1:100) and spectrin (Novocastra, 1:100). Primary antibodies were incubated on slides for 1 hour at room temperature.

2.4 *In silico* analyses

Crystal structures of the ABDs of dystrophin (PDB ID: 1DXX) [17], utrophin (PDB ID: 1QAG) [19], α-actinin 3 (PDB ID: 1WKU) [20], and fimbrin (PDB ID: 1AOA) [21] proteins were downloaded from Protein Data Bank database, while other protein information from UniProt database for dystrophin (UniProt ID: P11532), utrophin (UniProt ID: P46939), α-actinin 3 (UniProt ID: Q08043), and fimbrin (UniProt ID: P13797). Structural figures were prepared by PyMOL Molecular Graphics System (Version 1.3 Schrödinger, LLC). Structure-based sequence alignment was performed by using SALIGN web server [22]. Secondary structure predictions were performed by JPred4 server [23]. FoldX algorithm [24] and SDM web server [25] were used to calculate protein stability changes (ΔΔG, kcal/mol) upon point mutations using crystal structure of dystrophin ABD. Aggregation properties were predicted based on the structure of chain A of N-ADB by Aggrescan3D server using dynamic mode of prediction [26].

3. Results

3.1 Molecular genetic testing of *DMD* gene

Deletion/duplication testing of *DMD* gene by MLPA showed complete absence of the amplification product of probes hybridizing to exon 4 (Figure 2). PCR amplification of exon 4, however, did not confirm the deletion. Sanger sequencing of exon 4 revealed a novel point mutation (LRG_199t1: c.227A>T, LRG_199p1: p.Asn76Ile; submitted to LOVD database (https://databases.lovd.nl/shared/variants/0000171133) leading to the change of Asn residue to Ile at the 76th position (Figure 3). The mutation proved to be *de novo* being not detectable in the mother genomic DNA (Figure 3). In order to exclude the possibility of another potentially pathogenic mutation in the *DMD* gene, the whole coding region of the gene was sequenced. Data analysis of promoter, exonic regions, and +/-5 nucleotides from the

exon/intron boundaries revealed three hemizygous variants. Beside the unreported missense mutation c.227A>T, previously detected by Sanger sequencing, two other variants were found (both to be found in the Single Nucleotide Polymorphism Database (dbSNP) with >5% minor allele frequency (MAF), Table 1).

3.2 Muscle histology, dystrophin immunohistochemistry

In transverse sections the variation in fibre size was moderately increased due to the presence of scattered fibres which were either smaller than normal (atrophic) or moderately enlarged (hypertrophic). Occasional hypercontracted fibres were also detectable (a feature which is not pathological in biopsy specimens). The nuclei were predominantly in their physiological subsarcolemmal position. However, internalised nuclei were also noted in some parts of the biopsy affecting up to 8% of all fibres. The overall internal nucleation was not in the pathological range. There was no increase of the endomysial connective tissue. The blood vessels were normal. In small groups basophilic degenerative or regenerative fibres were detectable; such groups had approximately 30 fibres. Similar basophilic fibres were seen scattered amongst normal-appearing, mildly hypertrophic or atrophic fibres. The proportion of these fibres was not more than 1:50 (2%). Although these atrophic fibres were sometimes in perifascicular distribution, this was not characteristic feature in the biopsy and the findings were not those of dermatomyositis (where perifascicular atrophy is a prominent feature). There was no significant inflammatory cell infiltrate, although there were occasional mononuclear inflammatory cells, predominantly in areas rich in basophilic fibres. At the periphery of the biopsy occasional necrotic fibres were noted. No inclusions and no genuine vacuolisation was noted (there was mild freezing artefact in forms of small vacuoles, though) (Figure 4A). With special stains (PAS, PAS-diastase, Sudan-black) there was no evidence of abnormal glycogen or lipid accumulation. With ATPase reactions at 3 different pH (pH 4.2,

4.6, and 9.4) there was mild fibre type 1 predominance (which is not pathological in this muscle). The variation in fibre size affected the different fibre types equally. There was no evidence of fibre splitting. Cytochrome oxidase (COX) revealed occasional pale fibres and no COX negative fibres. There was no evidence of protein aggregates, subsarcolemmal increased staining intensity with this stain. With Gömöri's trichrome no features of mitochondrial myopathy (such as ragged red fibres) were noted and there were no inclusions (such as nemaline rods). With the NADH reaction the disruption of the myofibrillary architecture was noted in the degenerative and necrotic fibres; occasional dark fibres were also noted.

Dystrophin expression was analysed by means of immunohistochemistry using three antibodies targeting the centre (core), C-terminal and N-terminal region of the protein, designated as anti-dystrophin 1, 2, and 3 antibody, respectively. With antibody 1 there was variably intensity of sarcolemmal staining, segmentally severely reduced or lost. With antibody 2 there was intense, linear sarcolemmal expression consistent with normal staining pattern. With antibody 3 the majority of the fibres were negative, with segmental sarcolemmal expression in less than 5% of the fibres (Figure 4B, C, D respectively). The spectrin immunohistochemistry revealed normal, linear sarcolemmal expression. The α, β, γ, and d sarcoglycan showed linear sarcolemmal expression of moderate to high intensity, in occasional fibres with segmental decrease of expression. Merosin showed strong, linear, sarcolemmal expression (Figure 4F). All immunohistochemical reactions were performed parallel with the respective positive and negative controls (Figure 4E). The dystrophin immunohistochemistry has been repeated to confirm findings.

3.3 Predicting the effects of Asn76Ile mutation

3.3.1 Asn76 residue of human dystrophin

Structure of the N-ABD of human dystrophin was analyzed to explore the possible role of Asn76 residue in domain stabilization.

Based on the crystal structure, the Asn76 residue is located within the N-ABD, in helix E of CH1 subdomain (Figure 5A). According to published data, Asn76 does not constitute a part of any actin binding site (ABS) and is not involved directly in actin binding [17]. Side chain of Asn76 is not exposed to the surface of CH1 subdomain, rather it is buried between C and E helices and loop D. Side chain atoms of Asn76 are within hydrogen bond distance with main chain atoms of Asp46 and Glu65 residues in the crystal structure of N-ADB (Asn76.ND2-Asp46.O and Asn76.OD1-Glu65.N, respectively). These H-bonds may provide stabilizing interactions within the CH1 subdomain, making contact between helix E (Asn76), loop B (next to the N-terminal end of helix C) (Asp46), and loop D (Glu65) (Figure 5A).

3.3.2 Structure-based alignment of CH1 subdomain-containing proteins

Sequence alignment of human dystrophin, utrophin, α-actinin 3, and fimbrin protein N-ABDs showed high conservation of Asn76 residue (dystrophin numbering) (Figure 5C), in good agreement with the previously published analyses [17, 27, 28]. Conservation of Asp46 and Glu65 residues interacting with Asn76 was also investigated. The Asp residue (Asp46 according to dystrophin numbering) was found to be not fully conserved in the case of aligned CH1 subdomain-containing human protein sequences (Figure 5C), while 1 and 4 isoforms of α-actinin contain Asp residue, Asn can be found in this position in isoform 2 and 3 (data not shown). Residues corresponding to Glu65 of human dystrophin show the conservation of side chains having strongly similar properties (Asn, Asp, or Glu) in this position (Figure 5C).

3.3.3 Predicted effects of Asn76Ile mutation

Secondary structure prediction was performed using the sequence of p.Asn76Ile mutant N-ABD and showed no disruption of helix E, the mutant protein was predicted to maintain the overall secondary structural organization of the N-ABD of wild type.

Structure of the mutant human dystrophin protein (generated by FoldX) showed the loss of the conserved H-bonds of Asn76 with the neighboring Asp46 and Glu65 residues, due to the substitution of a polar Asn residue to a hydrophobic Ile. The mutation was predicted to be highly destabilizing ($\Delta\Delta G > 3$ kcal/mol) implying its possible association with protein malfunction.

The impact of p.Asn76Ile mutation on dystrophin aggregation propensity/solubility was also predicted. The analysis showed that both wild type (Asn76) and mutated (Ile76) residues are buried and are not part of aggregation-prone regions in CH1 subdomain.

4. Discussion

Here we report a case of a 14-months old male patient with markedly elevated creatine kinase level, mild hypotonia, and gross motor delay suggesting BMD/DMD. For the molecular genetic confirmation of dystrophinopathy a tiered genetic approach of the *DMD* gene was performed starting with MLPA analysis showing deletion of exon 4. Single exon deletions detected by MLPA always need to be confirmed by another method [29] and successful PCR amplification of exon 4 proved that our MLPA result must have been false positive. Sanger sequencing of exon 4 showed A-to-T transversion at the 227th nucleotide position lying directly at the 3' ligation site of the MLPA probe most likely leading to unsuccessful ligation and thus amplification. Sequencing of the entire coding region of *DMD* gene revealed no other potentially pathogenic variant except for the novel point mutation c.227A>T, previously detected by Sanger sequencing.

The c.227A>T variant was classified as likely pathogenic according to recently published criteria [30] upon the following: i) strong evidence is that the detected variant is *de novo* (the mutation was absent in the mother in a patient with disease and no family history), ii) moderate evidence is that it is absent from individuals in the 1000 Genomes Project and Exome Aggregation Consortium (ExAC), iii) supporting evidences are that the affected amino acid residue is evolutionary conserved between species and the patient's phenotype is highly specific for a disease with single genetic etiology. Markedly elevated CK levels and the clinical picture suggested dystrophinopathy. In order to further support pathogenicity of the detected variant and discriminate between BMD and DMD, muscle biopsy was performed.

The light microscopic examination revealed mild myopathic changes with regenerative activity. The expression pattern of dystrophin was pathological, with severely reduced and segmentally absent staining with one of the anti-dystrophin antibodies (dystrophin-1, targeting the core region of the protein), and with absent or severally reduced staining with dystrophin 3 antibody (targeting the N-terminal end of the protein). These findings are consistent with the *DMD* gene mutation revealed by the mutation analysis.

Although missense mutations are rarely detected in DMD/BMD patients, a major fraction of them occurs in the N-ABD of the dystrophin protein [31, 32].

Studies on human dystrophin are restrained by the problems of high-yield expression and purification of the full-length protein, therefore, studies mainly on the individual ABDs are published [33], and crystal structures of only the N-ABD, a WW- and EF-hand motif-containing fragment of the cysteine-rich domain, and the first spectrin repeat have been solved till now (last accession of PDB database: 12th June 2017). We also studied the dystrophin at the level of the N-ABD and used *in silico* methods to predict the effects of the detected point mutation on protein structure and stability, using the sequence and crystal structure of human dystrophin N-ABD. In addition, the predicted effects of p.Asn76Ile

mutation were compared to previously studied effects of p.Lys18Asn and p.Leu54Arg mutations [28, 33], being associated with DMD.

Not only sequence alignment (Figure 5C) but structural comparison of some CH1 subdomain-containing proteins (Figure 5B) also showed the conservation of Asn residue in this position (Asn76 according to dystrophin numbering). Similarly to the high conservation of Lys18 and the interacting Asn39 and Phe41 residues [33], our alignments also showed that the interactions of Asn76 with the surrounding Asp46 and Glu65 residues are conserved, suggesting the structural importance of these bonds in domain stabilization.

Substitution of Asn76 residue to Ile showed no impact on the secondary structure of helix E, and based on our predictions the p.Lys18Asn and p.Leu54Arg single mutants also share the secondary structure with the wild type ADB domain. This is in good agreement with the results of circular dichroism measurements, which indicated no alteration of global secondary structure of the ABD upon p.Lys18Asn mutation [33]. Extensive molecular dynamics simulations showed decrease or loss of helix I helicity in the case of p.Leu54Arg and p.Lys18Asn mutants, respectively, implying local conformational changes and long distance effects for the p.Lys18Asn and p.Leu54Arg mutations [28], but simple secondary structure prediction methods are not effective enough to predict the structural changes of the regions located distant from the site of p.Asn76Ile mutation. However, structural changes upon point mutations could be explored by experimental techniques or by extensive molecular dynamical calculations, these examinations were out of the scope and extent of this study.

Stability analysis showed highly destabilizing nature of p.Asn76Ile non-synonymous mutation. The p.Leu54Arg mutation was also predicted to have highly destabilizing nature ($\Delta\Delta G > 5$ kcal/mol), in good agreement with the previously considered effect of this mutation which affects the hydrophobic core of CH1 subdomain [31].

As p.Asn76Ile mutation has no effect on the secondary structure of ABD based on our predictions, it probably causes local conformational changes due to the disruption of stabilizing interactions within the CH1 subdomain. The mutation may alter the flexibility of loop B (due to the loss of Asn76-Asp46 H-bond between helix E and loop B) and spatial positions of loop D residues (due to the loss of Asn76-Glu65 H-bond between helix E and loop D), as well. Conformation of helix C could be also affected by the p.Asn76Ile mutation, because there is no direct hydrogen bond between helix E and helix C, only Asp46 located next to the N-terminal end of helix C interacts with Asn76 residue. Lys18 residue located in helix A of CH1 subdomain was also reported to stabilize the conformation of a turn in neighboring loop B, and its mutation disrupts the interactions between the residues of helix A (Lys18) and loop B (Asn39 and Phe41) leading to increased flexibility of loop B [33].

Although neither the wild type nor the p.Asn76Ile mutant ABD showed increased aggregation propensity of CH1 subdomain, interestingly, the most aggregation-prone human dystrophin models showed the highest root-mean-square deviation values for the residues of helix I and CH2 subdomain compared to the wild type protein. These results correspond to the changes observed previously by molecular dynamics simulations of Chakravarty and its coworkers, who found that p.Lys18Asn and p.Leu54Arg mutations compromise the helicity of helix I region and higher root-mean-square fluctuation values were observed for CH2 subdomain compared to CH1 [28]. This also indicates long distance effects for p.Asn76Ile mutation.

5. Conclusion

To predict the severity of the phenotype is challenging due to two facts. First, the detected likely causative genetic alteration is an unreported missense mutation. Second, the very early phenotypic observation of the patient resulted in an early genetic and pathological testing,

before the occurrence of the typical DMD/BMD symptoms. It is not possible to discriminate between BMD and DMD based on clinical features as the patient is only 4-year-old yet. *In silico* analyses of the protein structure supports the mutation pathogenicity but cannot provide information about disease severity. In this case, based on dystrophin immunohistochemistry results, we think that p.Asn76Ile mutation most likely leads to BMD, which diagnosis clearly needs to be re-evaluated over time on a clinical basis. As the mutation is a *de novo* point mutation for counselling purposes 2% recurrence risk has to be taken into consideration [16]. From the molecular genetic analysis point of view, our results highlight the importance of confirmatory testing of single-exon deletion MLPA results.

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Figure captions

Figure 1. Serum creatine kinase activity during follow-up time. Changes in the serum creatine kinase activity showed an increasing tendency over time. The grey zone indicates the reference range (24-195 U/L). CK, creatine kinase.

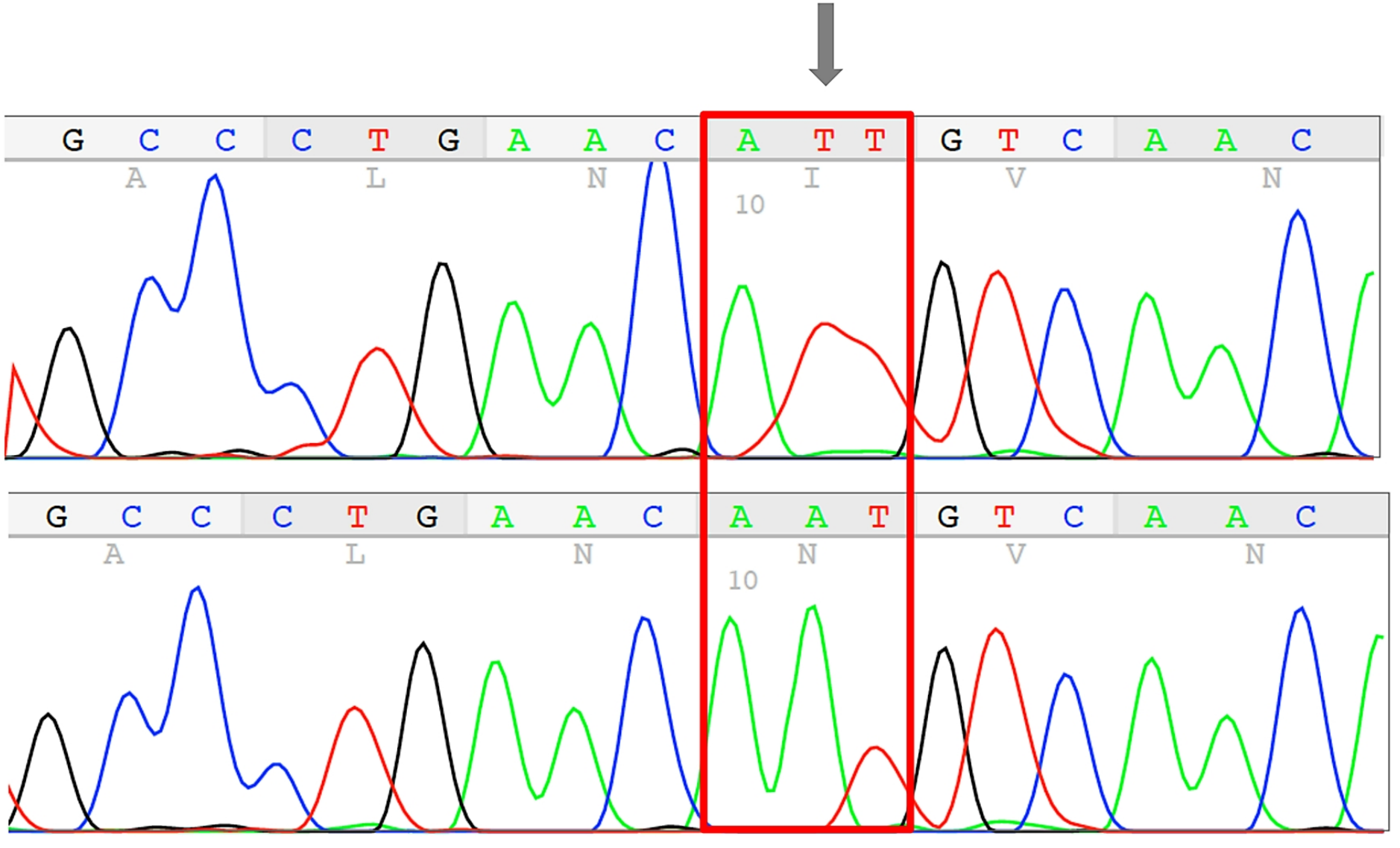
Figure 2. MLPA analysis of the *DMD* gene. Relative peak height values of probes hybridizing to exon 4 and two neighboring exons are shown. There was no amplification product of exon 4 in the patient DNA (light grey) sample (dark grey - normal control (male) DNA sample).

Figure 3. Sanger DNA sequencing of the *DMD* gene. A novel missense mutation (*arrow*, LRG_199t1: c.227A>T, LRG_199p1: p.Asn76Ile) was detected in the patient (upper panel) in exon 4. It proved to be *de novo* as it was absent in his mother (lower panel).

Figure 4. Muscle biopsy. (A) Increased variation in fiber size, internalized nuclei, few necrotic and basophilic regenerative fibers are seen. (B) There is reduced labelling with antibody recognizing the Dystrophin-1 epitope with segmental loss in numerous fibres. (C) Dystrophin-2 epitope shows normal linear sarcolemmal expression. (D) Dystrophin-3 epitope expression is noted only in occasional fibers with segmental distribution. (E) The negative control with omission of the primary antibody does not show staining. (F) The merosin expression is normal, with strong, linear staining of the sarcolemma.

Figure 5. Structure and sequence of human dystrophin, utrophin, α-actinin 3, and fimbrin proteins. A) Cartoon representation of ABD of human dystrophin. *Left panel*: antiparallel dimer formed by N-terminal ABDs. *Middle panel*: structure of the CH1 subdomain, the helix and loop numbering is indicated. *Right panel*: stick representation of Asn76, Glu65, and

Asp46 residues within CH1 subdomain; distances (Å) between the interacting atoms are indicated. B) Interactions of the conserved Asn residues in human utrophin, α-actinin 3, and fimbrin proteins. Regions corresponding to helix C, loop D, and helix E of human dystrophin are shown. Interacting residues are indicated by stick representation, residue names and distances (Å) are also indicated. C) Structure-based sequence alignment of actin-binding domains. Sequence numbering and secondary structural organization of human dystrophin is indicated above the aligned sequences. Helices and loops are labelled; Asp46, Glu65, and Asn76 residues (dystrophin numbering) are highlighted by green color.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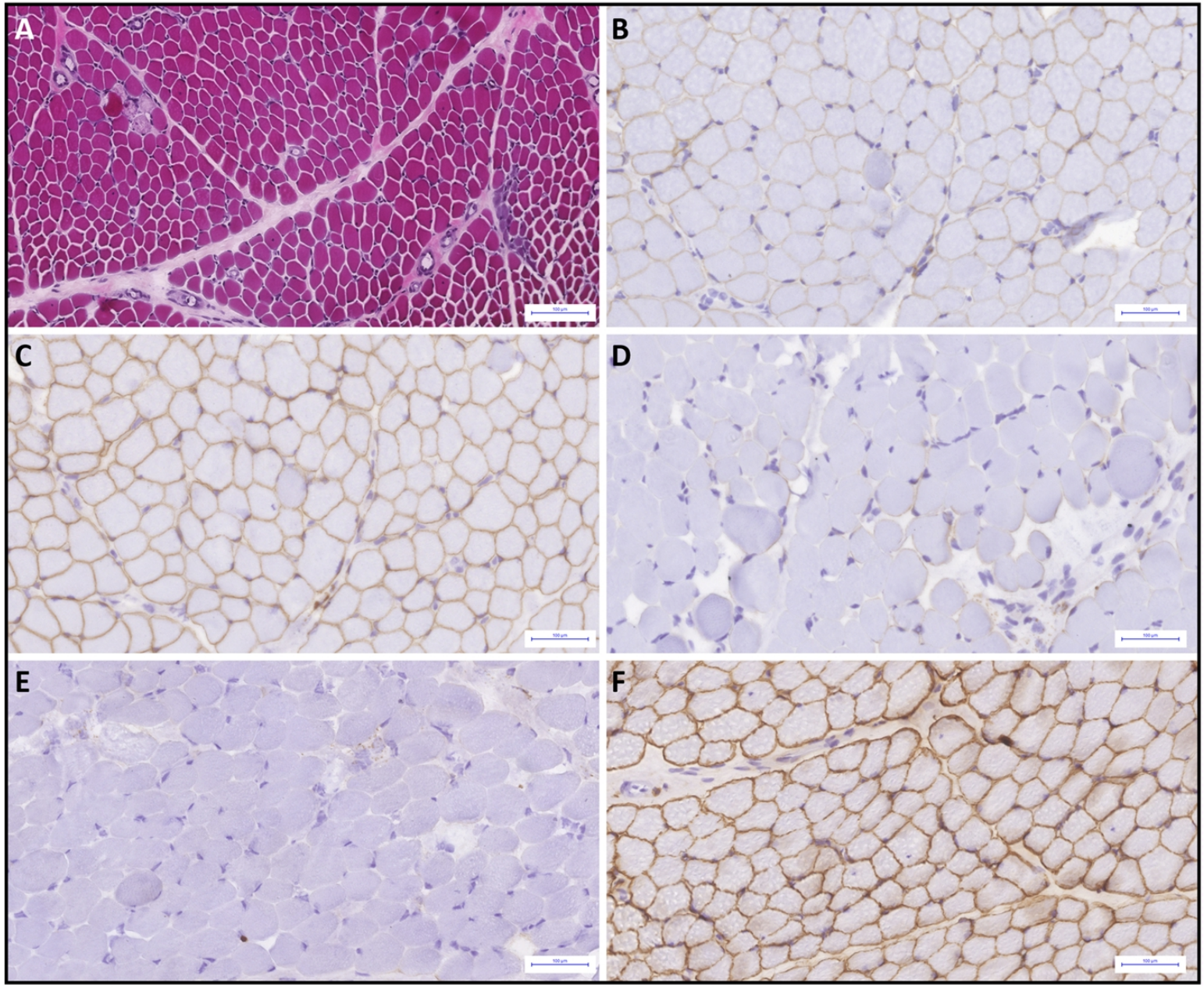
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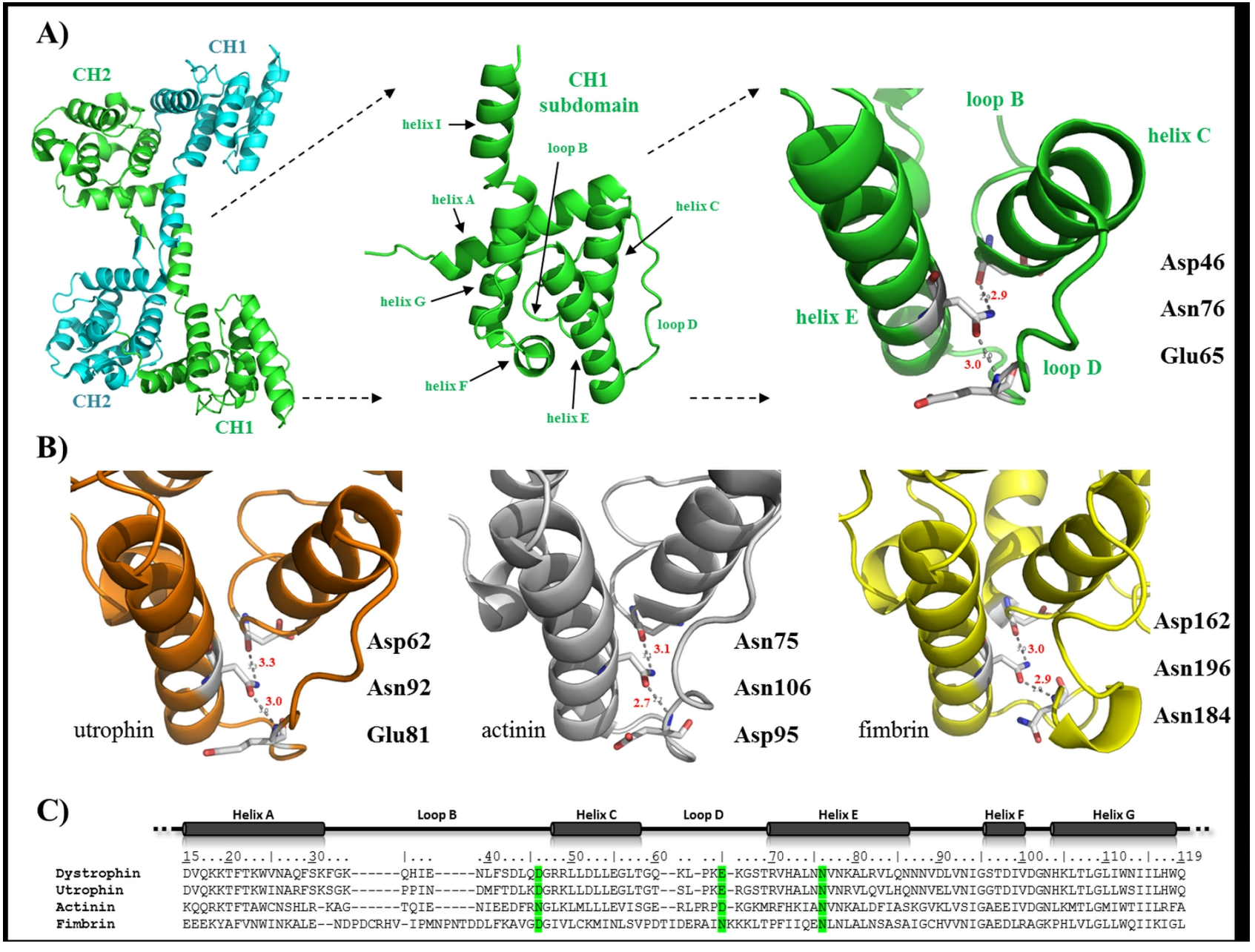
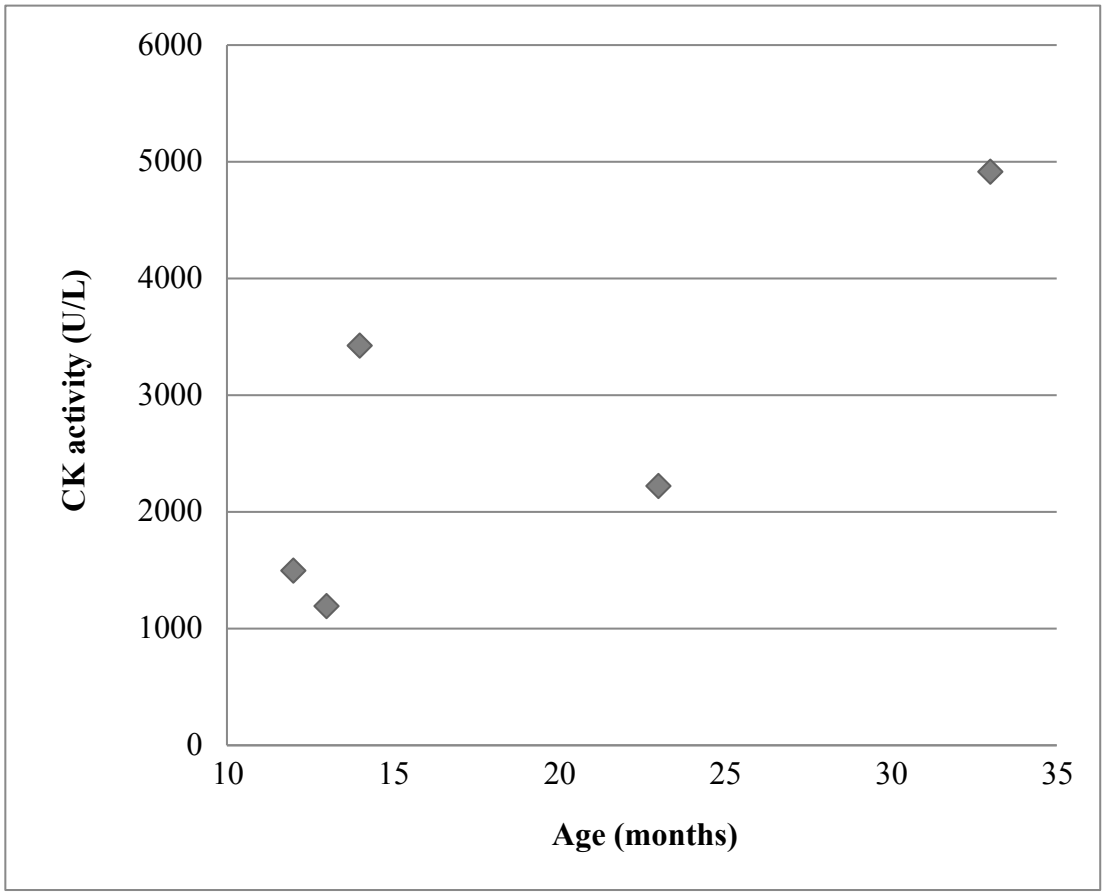

A)
CH1
CH2
CH2
CH1
CH1
subdomain
helix I
loop B
helix A
helix C
helix G
loop D
helix F
helix E
loop B
helix C
helix E
loop D
2.9
3.0
Asp46
Asn76
Glu65
B)
3.3
3.0
Asp62
Asn92
Glu81
utrophin
3.1
2.7
Asn75
Asn106
Asp95
actinin
3.0
2.9
Asp162
Asn196
Asn184
fimbrin
C)
Helix A
Loop B
Helix C
Loop D
Helix E
Helix F
Helix G
15...20...|....30... |... .40...|....50...|....60 .. ..| ....70...|....80...|....90...|....100..|....110..|...119
Dystrophin DVQKKTFTKWVNAQFSKFGK------QHIE-----NLFSDLQDGRRLLDLLEGLTGQ--KL-PKE-KGSTRVHALNNVNKALRVLQNNNVDLVNIGSTDIVDGNHKLTLGLIWNIILHWQ
Utrophin DVQKKTFTKWINARFSKSGK------PPIN-----DMFTDLKDGRKLLDLLEGLTGT--SL-PKE-RGSTRVHALNNVNRVLQVLHQNNVELVNIGGTDIVDGNHKLTLGLLWSIILHWQ
Actinin KQQRKTFTAWCNSHLR-KAG------TQIE-----NIEEDFRNGLKLMLLLEVISGE--RLPRPD-KGKMRFHKIANVNKALDFIASKGVKLVSIGAEEIVDGNLKMTLGMIWTIILRFA
Fimbrin EEEKYAFVNWINKALE--NDPDCRHV-IPMNPNTDDLFKAVGDGIVLCKMINLSVPDTIDERAINKKKLTPFIIQENLNLALNSASAIGCHVVNIGAEDLRAGKPHLVLGLLWQIIKIGL

Table 1. Detected variants in the *DMD* gene by pyrosequencing.

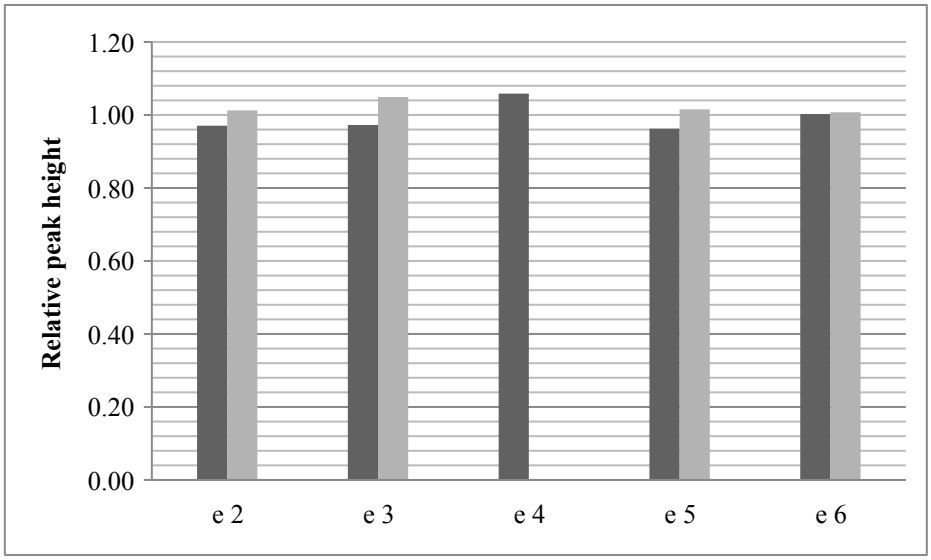
Localization	Genotype (hemizygous)[a]	Effect on protein[b]	dbSNP ID[c]	MAF[d]
exon 4	c.227A>T	p.Asn76Ile	-	-
exon 37	c.5234G>A	p.Arg1745His	rs1801187	0.47
exon 59	c.8810A>G	p.Gln2937Arg	rs1800280	0.12

Sequencing of the *DMD* gene revealed three hemizygous variants, two of them with a >5% MAF and an unreported missense mutation, c.227A>T. [c]dbSNP ID, single nucleotide polymorphism database identification number; [d]MAF, minor allele frequency, 1000 Genomes Project. Reference sequences: [a]LRG_199t1 and [b]LRG_199p1.

Age (months)	CK activity (U/L)
12	1497
13	1193
14	3425
23	2222
33	4915



	1	2
e 2	0.97	1.01
e 3	0.97	1.05
e 4	1.06	0.00
e 5	0.96	1.02
e 6	1.00	1.01



A novel point mutation affecting Asn76 of dystrophin protein leads to dystrophinopathy

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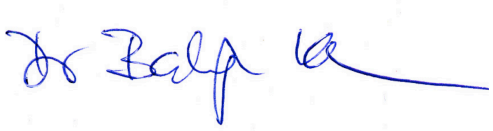
Author Agreement:

I, Dr. István Balogh, corresponding author declare, that all co-authors have read and agreed the contents of the paper. I sign this statement on behalf of all co-authors.

Debrecen, 27.06.2017



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